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STUDIES ON THE DEVELOPMENT OF *USTILAGO HORDEI* (PERS.) LAGERH.

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Studies on the development of Ustilago hordei (Pers.) Lagerh." submitted by Frank Kozar in partial fulfilment of the requirements for the degree of Master of Science.

Meinen Eltern

in dankbarkeit gewidmet

ABSTRACT

Biochemical mutants of *Ustilago hordei* were used to infect barley seeds in a study of the pathway of infection and the development of the parasite within the host. Histological studies confirmed earlier studies indicating that the parasitic mycelium was not confined to the nodes, internodes, spikes and flag leaves of the host plant, but was present also in the growing points of infected tillers. These histological results were confirmed by studies in which the parasite was recovered from pieces of host tissue, identified and grown *in vitro*.

Further, cultures extracted in this way frequently dissociated so that re-identification of the original marked nuclei was possible. This served not only as a direct confirmation and extension of the histological observations, but also as a means for revealing evidence of mitotic recombination. The results of this study strongly suggest that mitotic recombination does in fact occur in *Ustilago hordei*.

Evidence was also obtained that one of the recovered cultures was a diploid capable of infecting the host.

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INTRODUCTION AND LITERATURE REVIEW

Smut in barley may be caused by three different species of Ustilago. Separation into three species is based in part on the mode of infection by the parasite and on the disease reaction of the host. Ustilago hordei (Pers.) Lagerh. is a seedling-infecting covered smut. Ustilago nigra Tapke is a seedling-infecting loose smut, while Ustilago nuda (Jens.) Rostr. is an embryo-infecting loose smut. The development of U. nuda in germinating barley and wheat and in the subsequent plant has been described by a number of investigators. Batts and Jeater (1958) investigated the path of Ustilago tritici infection in the developing wheat grain. Malik and Batts (1960) published a detailed account of the mode of infection in barley and the production of teliospores in the spike. They found that their views on the infection of barley by U. nuda were in agreement with the one stated by Pedersen (1956). Gage (1927), who investigated the pathway of infection of Ustilago avenae, found, contrary to Butler's (1918) observations, long, unbranched hyphae sparsely scattered throughout the internodes and short, branched hyphae in the nodes. Wang (1934) investigated spore formation in U. avenae. These and other conflicting reports prompted Mills (1966) to re-investigate the path of infection and to study the development of the mycelium and spore formation of U. avenae in panicles, leaves and culms of the host.

Batts and Jeater (1958) found that in infected embryos the mycelium after penetrating the pericarp of Hordeum vulgare passed through the parenchyma along the integuments and entered the scutellum and the growing point. It infiltrated most parts of the embryo. After germination

of the seed the mycelium was carried to the first crown node by the elongation of the epicotyl. As growth continued mycelium was carried up in the young spike by the elongation of the internodes of the stem. In its early development the spike was infiltrated by tangled hyphae which then aggregated in the florets to be ultimately replaced by spores formed by hyphal segmentation.

Mills (1966) in his study of U. avenae found direct promycelial penetration of coleoptile and first internodes. Intracellular mycelium was present in coleoptile and first internodes, while in the first leaf and deeper tissues it was mainly intercellular. After reaching the growing points and internodes it was carried upward as elongation took place.

Details with respect to the development of U. hordei in the host are rather scanty. Grasso (1952) according to Fischer and Holton (1957) "described and illustrated the pathological histology of sporulation of U. hordei on the upper portion of the culms of barley." More recently, the sporidia of U. hordei have been successfully recovered from the growing points of infected tillers (Neilsen, 1967).

The study of smut merits investigation from the standpoint of the agricultural industry; U. hordei also has much to offer as a tool for genetic investigation because:

(a) it possesses a normal sexual cycle, facilitating standard genetical analysis;

(b) its pattern of growth enables the investigator to employ the usual bacteriological techniques;

(c) its haploid sporidia are readily cultured *in vitro* and may be kept as such for indefinite periods of time. They may be readily stored in silica gel for future use (Perkins, 1962);

(d) the organism is fast growing;

(e) because it lacks a complex fruiting body the environment of its meiotic cells may be readily altered to meet specific requirements.

This study deals with the course of development of *U. hordei* in the barley host with the aim of determining whether it follows the pattern of development of *U. nuda*, *U. tritici* and *U. avenae*. Moreover, using a modified technique of sporidial extraction (Neilsen, 1967), individual sections of the host culms and growing points were studied to determine the growth behavior of the parasite.

After the recovered sporidia were grown *in vitro*, they were identified and used to re-infect the host. A genetic analysis was carried out to study the haploid, diploid and dikcaryotic stages of the organism.

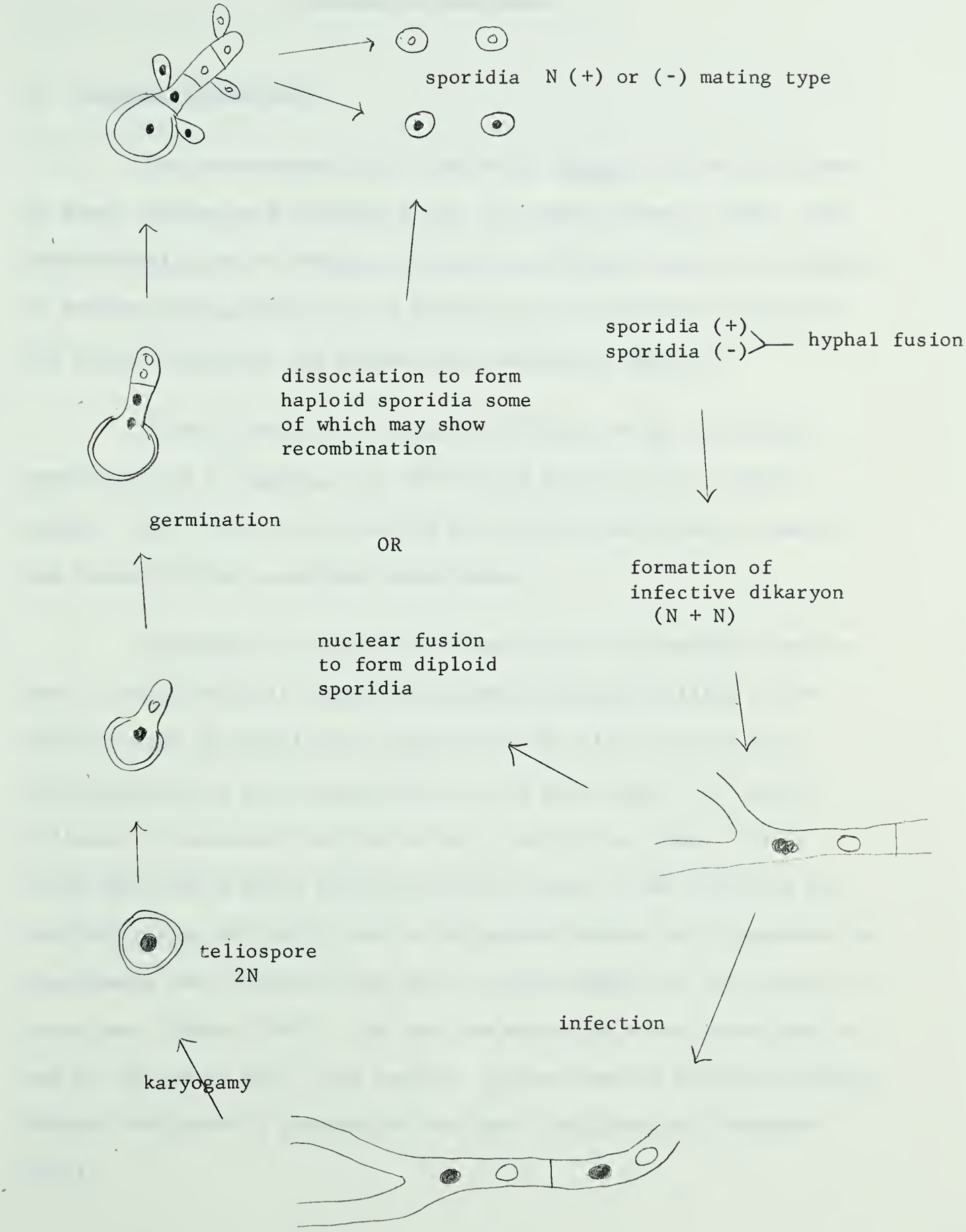
The studies of Grasso, Malik, Batts, Jeater and Mills (loc. cit.) employed histological techniques to investigate the course that the parasite takes as the host matures. In this study histological findings were checked against genetical results through the use of nuclei labelled with biochemical markers.

The Life Cycle of U. hordei

The life cycle of U. hordei consists of an infective parasitic phase and a non-parasitic phase. The latter begins with the germination of the teliospores. Hüttig (1931) found that upon germination the diploid nucleus moves into the promycelium and divided mitotically. It then forms a crosswall. The next division, a meiotic one, produces a four-celled septate promycelium (Hüttig, 1931; Wang, 1934). Today it is generally considered that the first division is a meiotic one, giving a reduction in the chromosome number from 2N = 4 to N = 2; the second division, a mitotic one, leaves the chromosome number unchanged. The promycelium then buds off numerous haploid sporidia (Wang, 1934; Holton, 1936a). In favorable circumstances these sporidia immediately fuse to form the dikaryotic (n + n) phase which initiates the infection and which persists in the infected plant until teliospores are formed again. This infection may take place within the embryo so that the embryonic nodes, internodes and spike (as they grow upward) carry masses of mycelium with them.

Dickinson (1927) showed that penetration of the seedling in its very early stage of growth is by fusion hyphae which resulted from the union of two compatible sporidia.

Prior to spore formation, the mycelium breaks into segments, the conjugate nuclei fuse and the teliospores are formed. Wang (1934) reported that in culture some hyphae shortened and developed a thick membrane enclosing two fused nuclei.



Text-figure 1. Schematic diagram of life cycle of U. hordei

MATERIALS AND METHODS

A. Biological Materials

The two monosporidial lines of U. hordei (I_4+ and E_3-) used in Bauch testing were provided by Mr. P. Thomas (Thomas, 1965). The experimental plots of Vantage, Hannchen and Odessa varieties of barley at Parkland Farm, University of Alberta, were the source of many of the plants from which the mutants were recovered, Table I.

Seeds of Hannchen, Vantage and Odessa, which are fairly susceptible to U. hordei, were chosen from the plots for further study. Since Odessa barley showed the greatest percentage of smut it was chosen for the greenhouse experiments.

To reduce the chance of contamination with foreign inoculum and to loosen the hull around the caryopsis (thereby aiding in the effectiveness of inoculation) Odessa seed was first treated with formaldehyde (one part formaldehyde to 320 parts water for one hr) followed by subsequent washing in water and drying (Tapke, 1942). Since there was a heavy infection of loose smut on the barley in the Parkland plots, all seeds used in the growth chamber and in germination experiments were treated using the hot water method for the control of loose smut (Tapke, 1942). The seed was heated in a hot water bath for one hr, 35 min at 49° C, and cooled. It was then run through a Waring Blendor and dried in preparation for later use (Popp and Cherewick, 1953).

Table I. Mutants of *U. hordei* used in this study*

Mutants	Description
Ad X29	Adenine mutant, mating type (+)
Arg V240	Arginine mutant, mating type (+)
Arg V240	Arginine mutant, mating type (-)
Arg V244	Arginine mutant, mating type (-)
Iso V13	Isoleucine mutant, mating type (+)
Leu V394	Leucine mutant, mating type (-)
Leu V419	Leucine mutant, mating type (+)
Meth V364	Methionine mutant, mating type (-)
Morph X73	Morphological mutant, mating type (-)
Pan V191	Pantothenic acid mutant, mating type (+)
Pan V359	Pantothenic acid mutant, mating type (+)
Pyrid V26	Pyridoxine mutant, mating type (+)
Pyrid V26	Pyridoxine mutant, mating type (-)

* These mutants were isolated by Dr. C. H. Hood, University of Alberta, 1964-66. Different mutants for the same requirement represent independent isolates from different experiments and are therefore considered to represent different mutants at one locus or several loci.

B. Mating and Host Inoculation

The methods used and described by Thomas (1965) were employed in preparing compatible sporidial cultures and inoculating these on the barley seed. The required number of 125 ml aluminum foil covered Erlenmeyer flasks each containing 50 ml of complete medium were inoculated with fresh sporidial material taken from agar plates (Table I). The flasks were placed in the shaker-incubator for 24 hr at 22° C (± 2° C) for 24 hr..

Approximately 100 formaldehyde-hot water-treated seeds were placed in vials and covered with inoculum. The seeds were subjected to the vacuum method of inoculation, 55 cm Hg for 25 min (Tapke, 1942). This method was used in preference to the suspension method of Tapke (1942), since it was felt that the sporidia would be carried beneath the hulls and be provided with a more favorable environment upon germination. The sporidia were drained off and the seeds were left in vials for one hr in the hope that this moist condition would favor sporidial germination, penetration and infection. The seeds were then placed in coin envelopes and dried for five days before planting.

C. Histological Studies

For examination of plants showing infection, specimens from the experimental plot were selected. Sections of plants were fixed in Formalin-Alcohol-Propionic acid (90:5:5) for 24 hr and stored in 70% alcohol for later examination (Johansen, 1940).

Three methods of preparation for examination of these specimens were employed:

(a) Surface preparations. These preparations were used mainly to study the early stages of fungal development in inoculated kernels, which had been germinated in sterile Petri dishes in an incubator at 22° C. A number of constituents of the kernel (hull, pericarp, embryo, etc.) were separated and examined by mounting in cotton blue in lacto phenol (Darlington and LaCour, 1950) after clearing in cold potassium hydroxide (10%) for six hr.

(b) Squash preparations. This method was employed with soft tissues (i.e. coleoptiles, spikes, young tillers, etc.) where quick verification of the infection was imperative. The material was stained with cotton blue in lacto phenol.

(c) Microtome sections. Approximately 2,000 longitudinal and transversal serial sections (10 to 15 microns thick) of the plants at various stages of development (i.e. nodes, internodes and spikes as well as leaf blades) were made and examined. Some of these were stained with thionine blue and counterstained with Orange G (Conn, Darrow and Emmel, 1962) while others were stained with Johansen's Quadruple Stain (Johansen, 1940).

D. Sporidial Studies

In an attempt to recover sporidia of U. hordei from the infected host prior to teliospore formation, sections of nodes, internodes, secondary roots, crowns and spikes from plants infected with known mutants were placed in shake cultures. Much difficulty was experienced with contamination, both bacterial and fungal. To overcome this problem two procedures were followed:

(a) After being shredded longitudinally with scissors sections of material were washed in a 5% solution of sodium hypochlorite for 30 seconds after which they were washed in several changes of sterile distilled water before transfer to shake cultures.

(b) Sections were dipped in 95% alcohol and flamed before being placed in shake cultures.

Although culturing was carried out under standard sterile procedures and penicillin-streptomycin (50 international units per ml of each medium) was added to the culture medium, some contamination resulted.

(i) Media

Shake cultures of complete medium consisted of 5 g Difco yeast extract, 5 g salt-free casein hydrolysate (N.B. Co.), 10 g dextrose, 50 mg tryptophane, 20 ml Vogel's (1956) modified salt solution, 10 ml vitamin solution (Holliday, 1961) and 1 liter distilled water. The medium was autoclaved for 10 min at 115° C. For agar plates 2% Difco bacto was added.

Minimal medium contained 20 ml Vogel's salt solution and 10 g dextrose per liter of distilled water. Mating tests were performed on Bauch plates which contained 20 ml Vogel's salt solution, 2 g dextrose, 20 g Difco bacto agar per liter of distilled water.

Supplemented agar medium was prepared by using minimal agar medium with the specific requirements added, i.e., 100 µg of amino acid per ml (Holliday, 1961).

All shake cultures were maintained in aluminum-foil covered Erlenmeyer flasks placed in a New Brunswick incubator-shaker Model R27 and incubated at 22° C (± 2° C). Isolated cultures and I_4+ and E_3- stocks were maintained on complete medium agar and stored at 4° C.

(ii) Replica Plating

Recovered sporidia were taken from the shake cultures under sterile conditions when the concentration of sporidia approached 10^6 - 10^7 per ml. To ensure approximately 100 colonies per plate sporidial counts of the dilutions of the original cultures were established with a Spencer hemocytometer.

The agar media were incubated for either three or four days at 22° C and 16 colonies of each group of recovered sporidia were transferred to complete agar medium on gridded plates. These were incubated for five days at 22° C. The colonies were then replicated on to complete agar, on to minimal agar and on to each of the required supplemental agar plates. Replication was carried out using the velvet press method according to the technique of Lederberg and Lederberg (1952).

Replicated agar medium plates were placed in the incubator for four or five days before being examined to determine the biochemical characteristics of the mutant recovered. The mating type of each colony was determined by means of the Bauch test (Bauch, 1927, 1932; Fischer and Holton, 1957)(Appendix 2).

E. Photography

Photographs of the barley plants and spikes were taken with an Asahi Pentax, Model SI, 35 mm camera. For photographing the spikes, extension tubes were employed. Panatomic X film was used. A Leitz Ortholux microscope equipped with a Leitz 4 x 5 in camera was used for photographic recording of sporidia and serial sections. The film, Contrast-process Ortho sheet film, was developed in D-11 developer and printed on a contact printer.

F. Seeding

After infection, seeds were grown in pots in a growth chamber (February, 1967). Eight seeds were placed 3/4" deep in each of 50 pots containing California Soil Mix (Baker, 1957). Ten pots each containing ten noninfected seeds served as controls. The temperature was set at 20° C ($\pm$ 2° C) for germination and at early stages of growth this was increased to 24° C. The length of day was regulated from 8 hr of light for germination and early stages of growth to 10 hr for later growth and ripening. The plants were fertilized as required and to enhance ripening a phosphate fertilizer (one tablespoon of 15-30-15 per gal of water) was applied six weeks after germination.

G. Determination of Haploidy and Diploidy of Sporidia

Several methods of determining DNA contents of sporidia were employed. In the first method (Schneider, 1957) the nucleic acids were separated from sporidia by preferential solubility in hot trichloroacetic acid. Upon isolation the nucleic acids were quantitated by colorimetric means (Appendix I).

The second method involved staining the sporidia with Feulgen and using microphotometric evaluation of the cytochemical color reaction of the Feulgen on the DNA (deoxyribose nucleic acid) of the nucleus (Patau, 1952).

RESULTS

A. General

In many of the smutted barley spikes collected at the University of Alberta Parkland Farm during a two week period in August, 1966, following a rainy and cool period a condition which will be referred to as "clubbing" occurred. The clubbed spikes had a stunted appearance and showed a marked reduction in teliospore formation and number compared to other smutted spikes collected before and after this period.

Smutted spikes were found to emerge a little later than normal spikes and often showed deformities of emergence. The diseased spikes displayed varying degrees of twisting, bending and curling of the stem (Plates 1 and 2).

According to Faris (1924) the amount of smut on individual spikes varies greatly. This was found to be the case in this study also (Plate 3). As a general rule in awned varieties of barley the awn on the upper portion of the floral bracts was present. The lower portion of the membrane was semi-transparent revealing the enclosed teliospores. In the majority of cases the complete spike was smutted. The remainder were only partially smutted, the clubbed spikes showing very little smut (Plate 4). In almost all instances it was the lower portion of the spike which bore the teliospores.

Shortly after Faris' observation of sporulation in the vegetative tissues of the host Rump (1926) observed sporulation of *U. hordei* in the nodes of the host plant. In the author's study teliospore formation was observed in the fourth node (Plate 10).

Studies by Roberts (1967) indicate that there is variation in infection and that some tillers of infected plants may be unaffected by the parasite. This was also found to be true in re-infection studies of this report. The results of this study suggest that there is variation in the amount of mycelium present in the infected embryo. Thus in embryos which have small amounts of mycelium present only a limited number of the tillers may become infected. In Roberts' study, Odessa was found to be very susceptible and a large percentage of the tillers showed smut. It would therefore appear that this variety provides the proper conditions for the development of large quantities of mycelium.

C. Histological Studies of the Infection Pathway

From a study of germinating seeds it was found that germinating spores were prevalent on the hull of Odessa barley at 24 hr (Plate 5). After 48 hr few germinating spores remained, and a large amount of intercellular mycelium, some of which had penetrated through the pericarp, was present. At approximately 72 hr the hull was profusely covered with mycelium and the epidermal wall was infiltrated by intercellular mycelium. At 100 hr intracellular and intercellular mycelium were observed in the embryo, i.e. in coleoptile and node primordia. No mycelium was seen in the control seeds.

Examination of approximately 2,000 serial sections of crowns, nodes, internodes, growing points, spikes and flag leaves of infected and control tillers provided evidence for the pathway of infection. Both of the staining methods used indicated the presence of mycelium in the crown, internodes, nodes and spikes. The septate hyphae were of

two types: long, slender and branched, and short, thick and branched. Both intercellular and intracellular types were present (Plates 6, 7, 8). The form of the mycelium varied with the tissue in which it was located. One infected tiller studied in detail from crown to spike had mycelium present in the crown, the nodes, the internodes, the flagleaf and the spike. In the crown and nodes the short, thick and branched mycelium was concentrated in the meristematic tissue (Plates 6, 8) in contrast with earlier studies on the development of U. tritici and U. nuda (Batts and Jeater, 1958; Malik and Batts, 1960) which showed that the mycelium of U. tritici and U. nuda was confined to the internodal and spike regions. The cytological and biochemical results of this study indicated that mycelium of U. hordei was present in the internodal regions as well. In the internodal regions mycelium was mainly long, slender and relatively unbranched as it forced its way between the cells (Plate 8). In this respect there is a striking similarity between U. hordei and U. avenae.

Masses of teliospores occasionally appeared on the flagleaf (Plate 1). Staining of preparations with cotton blue indicated that sporiferous hyphae were present in the mesophyll of the leaf. These aggregated to produce pockets of teliospores, which, as they matured, ruptured the epidermis.

In one of the infected tillers teliospores were observed in cross sections of the fourth node and of the stalk immediately below the spike. Serial sections of the fourth node showed that these consisted of pockets of teliospores situated mainly in the upper portion of the node in parenchymatous tissue (Plate 10). In other sections a

few pockets of teliospore production took place in a series of neighbouring cells by segmentation of intracellular mycelia. The process of spore formation began with the thickening and segmentation of the intracellular mycelium. The mycelial segments enlarged and the protoplasm became so dense as to obscure the nucleus. As the maturation process continued the enlarged segments began to round off and the teliospore wall was formed. The oldest spores were always situated in the center of the spore pockets.

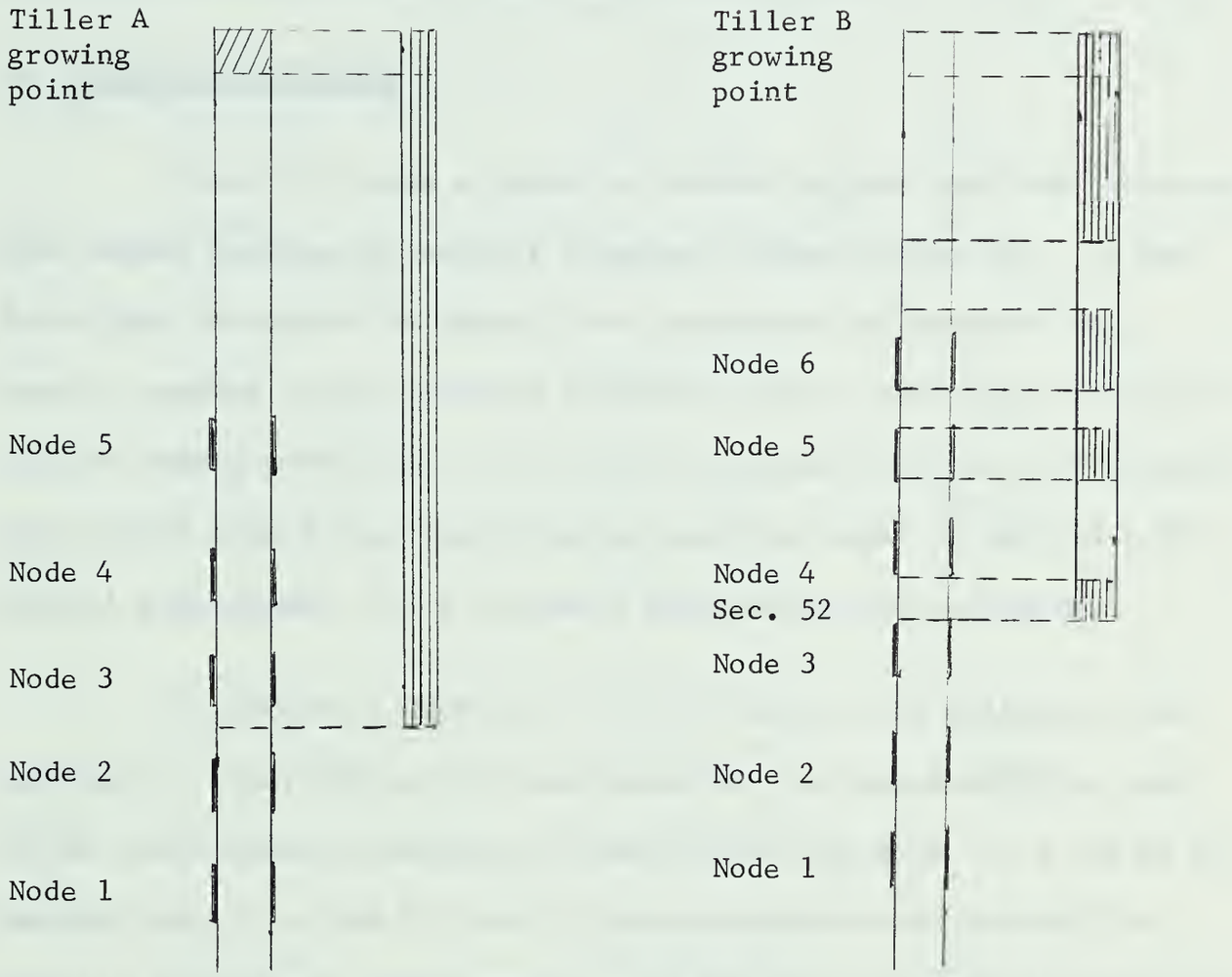
The formation of teliospores in the spike followed a pattern similar to that found in the nodes. Teliospore formation took place while the spike was still in the developmental stage. By the time the infected spike had reached a length of about one centimeter teliospores were present and the entire spike had been infiltrated by sporiferous mycelium and took on a creamy yellow color. At emergence the kernels had been replaced by black masses of teliospores.

In one preparation of mycelium grown in vitro and stained with Feulgen's stain clamp connections were observed. The presence of these was confirmed by Person (1967).

C. Biochemical Studies of the Infection Pathway

The successful extraction of sporidia from serial segments from infected selfed tillers of barley confirmed the histological data with respect to the presence of mycelium. Text-figure 2, which shows the areas of the two tillers from which sporidia were recovered, indicates that sporidia were recovered from nodes, internodes and flag-leaf. Both mating types were recovered in all but one site. Of the

Text-figure 2



Text-figure 2 shows areas from which mutants were recovered from two tillers that had been infected by V_{26} Pyr (+) x V_{26} Pyr (-). The tillers were approximately 60 cm long. The recovery time varied from three days to six weeks. The striped regions indicate those areas from which sporidia were recovered.

16 colonies tested from this recovery only one mating type (+) was identified. Three sections of the tiller produced sporidia which grew on minimal medium and gave indefinite Bauch tests.

D. Reinfection Studies

Table III shows a number of marked haploid sporidia recovered from randon sections of several infected tillers (Table II). It was found that the number of plants that germinated and survived was greatly reduced in pots showing infection. The plants with no smutted tillers showed germination and survival to maturity of up to 90%, while those which showed heavy smutting had survival rates of only 20 - 50%. Control pots showed 92% of the seeds germinating and surviving.

To determine whether or not the smut in the tillers in the reinfection study (Table III) was caused by the marked haploids used in the inoculation teliospores of two crosses 664 Meth (-) x 104 Ad (+) and 664 Meth (-) x 824 (Z) Iso (+) were germinated and checked for specific nutritional requirements. The 664 Meth (-) x 824 (Z) Iso (+) cross yielded 13 wild-type colonies and three methionine among the 16 colonies checked. Both mating types were present. Germination of these teliospores was very poor. The 664 Meth (-) x 104 Ad (+) cross yielded seven Adenine, two Methionine, seven wild type and four Adenine-Methionine mutants among the 20 colonies checked. Both mating types were recovered.

Table II. Data of mutant sporidia recovered from random sections of infected tillers

Row no.	Inoculum	Mating type	Barley section from which sporidia were recovered	Mutant recovered	Mating type
62	Pan V191	+	Third node	Pan	+
	Arg V244	-	Third node	Arg-Pan	+
104	Ad X29	+	Node below spike	Ad	-
	Morphological X73	+	Node below spike	Morph.	-
104	Ad X29	+	Second node	Ad	+ & -
	Morphological	-	Second node	Morph.	-
104	Ad X29	+	Third node	Ad	+ & -
	Morphological	-	Third node	Morph.	-
659	Pan V359	+	Growing point	*	
	Arg 240	-	Growing point	*	
664	Arg V240	+	Growing point	Not recovered	---
	Meth V364	-	Growing point	Meth	-
824(1)	Iso V13	+	Growing point	*	
	Leu V394	-	Growing point	*	
824(2)	Iso V13	+	Growing point	Iso	+
	Leu V394	-	Growing point	Not recovered	---
824(3)	Iso V13	+	Growing point	*	
	Leu V394	-	Growing point	*	
824(Z)	Iso V13	+	Node no. 5	Iso	+
	Leu V394	-	Node no. 5	Not recovered	---
872	Leu V419	+	1/4" growing point	Leu	+
	Arg V240	-	1/4" growing point	Not recovered	

* Recovered sporidia grew on all the media tested including minimal.

Table III. Re-infection studies showing recovered marked haploids used

(a) Greenhouse

Recovered mutant used on seeds	Number of smutted tillers
664 Meth (-) x 62 Arg Pan (+)	2
664 Meth (-) x 824 (Z) Leu (+)	6
664 Meth (-) x 104 Ad (+)	11
872 Leu (+) x 104 Ad (-)	2
Recovered mutants used on seedlings	
664 Meth (-) x 62 Arg Pan (+)	2
664 Meth (-) x 824 (Z) Leu (+)	1

(b) Experimental plots

The data below shows the results of re-infection studies at Parkland plot, May - August, 1967.

Recovered mutant used on seeds	Estimated % of smutted tillers
664 Meth (-) x (I) 872 Leu (+)	20
664 Meth (-) x 824 (Z) Iso (+)	20
664 Meth (-) x 62 Arg-Pan (+)	30
664 Meth (-) x 62 Pan (+)	15
104 Ad (-) x 824 (Z) Iso (+)	5
104 Ad (-) x 104 Ad (+)	clubbed
824(3)	1*

* 824(3) was chosen at random from a number of recovered sporidia (i.e. 659, 824(1) Table I and section 52 of Tiller B Text-figure 2). It was suspected of being a diploid since it grew on minimal medium and contained approximately twice the DNA as compared to a known haploid. Infection by 824(3) of the host would indicate that it is a diploid.

E. Results of Determination of Haploidy or Diploidy of Sporidia

When the suspected diploid sporidia of Row 824(3) were compared with the known haploid 824 (Leu 394) the following observations were made:

(1) cultures of 824(3) grew on minimal plates, 824 did not

(2) cultures of 824(3) showed indefinite Bauch tests

(3) DNA assay indicated approximately double the DNA content in 824(3) compared to 824 (Leu 394)

(4) results of Feulgen staining revealed the presence on only one nucleus thereby ruling out the dikaryotic condition in 824(3)

(5) barley infected with sporidia from 824(3) produced smut.

DISCUSSION

Although comparatively few investigations on the development of the established parasite in the barley host have been reported on, a number of generalizations can be made. Date reported here are in agreement with Dickinson (1927) with respect to the manner in which the sporidia gain entrance to the seed embryo. Squash preparations indicated that after germination the sporidia gained entrance to the developing embryo by means of fusion-hyphae which worked their way through the hull and the aleurone layer. Mills (1966) working with U. avenae found penetration holes and sheathing through which the invading hyphae entered. In U. hordei after the formation of the promycelium the resulting hyphae, largely intercellular, forced their way through the hull and aleurone layer. Subsequently, the mycelium found its way towards the embryonic region, an area of actively dividing cells. No mycelium was found in the endosperm.

The invading mycelium proliferates in the embryonic tissue and resides in the growing point pirmordium, node initials and internodal regions as the growth of the tiller begins. This may be an important stage in the establishment of the parasite in the host. If the host is slow in sending forth its shoot, the parasite may be afforded an opportunity for proliferation since the conditions of growth may be ideal. As the seedling continues its growth, the expansion of the stem starts at the lowest internode of the tiller which is first to elongate. It carries with it any mycelium present in the particular structure in the embryo, until all of the nodes of each tiller are formed. Once the nodes and internodes approach the termination of their

growth the parasite still continues its growth and development within the confines of the meristematic tissue. There are two mechanisms of invasion and proliferation employed by the fungus: that is to say: (i) growth in the meristematic tissue and (ii) transportation within the upward moving tissues of the elongating stem. Both mechanisms serve to distribute the mycelium throughout the length of the host. As the host matures the amount of meristematic tissue diminishes. It may well be that the reduction of the amount of meristematic tissue brought about by the maturation process seriously curtails the food supply of the parasite. Sporulation may be a mechanism employed by the parasite in its struggle for survival. The hyphal segments thicken, break up and form spores.

The mycelium which was present in the growing points of the developing spikes similarly is carried upward in the tiller. As the spike is carried upward the mycelium present has the opportunity of developing for a relatively long period of time since the growing point matures relatively late and its meristematic tissue is well supplied with nutrients. By the time the spike begins the maturation process its tissues have been invaded by large masses of mycelium. These may become sporiferous and eventually form teliospores probably due to the diminishing food supply in the host.

Differences in the form of the mycelium may well be determined by the nature of the tissue in which it is situated. The resistance offered by the different cells may determine the amount of intercellular space available to the invading hyphae and thus a modification of hyphal form may result. In meristematic tissue the hyphae shorten, thicken

and branch out. Short, thick hyphae, some of which may have been haustoria (Plate 6) were observed in parenchymous cells, whose walls could be relatively easily penetrated. In contrast the firm walls of sieve tubes may have forced the parasite to send forth slender hyphae which followed intercellular spaces (Plate 8).

The detection of haustoria in the nodal areas marks another difference between the development of Ustilago hordei and U. tritici, U. nuda and U. avenae. Neither Batts (1958) nor Mills (1966) mention these structures in the development of U. tritici, U. nuda and U. avenae. Since these structures were detected mainly in the parenchymatous tissue, it might very well be that these highly modified hyphae serve as a possible pathway for infiltrating the host cell.

The reduction in teliospore numbers in the clubbed spikes may have been due to a temperature effect. Kavanagh (1960) with respect to loose smut stated: "reduced chlamydospore formation at high temperatures is due largely to the direct effect of high temperature in reducing the growth and vigor of the fungus" (page 188). This may explain the appearance of large numbers of clubbed spikes at the Parkland plots after a period of hot weather, which followed a two-week, cool and rainy period. There may well be other factors involved, however, since one plant in the re-infection study even though grown in the growth chamber (where temperature conditions were relatively uniform), showed the clubbing effect.

Results from Table II i.e. 659; 824(1); 824(3) and from Text-figure 2 i.e. section 52 of tiller B indicate that diploids may be recovered along with the haploids, but at a lower frequency. This indicates that two unlike haploid nuclei in a dikaryon may fuse to form a heterozygous diploid nucleus, which then continues to multiply in the same tissue or culture along with the haploid nuclei of heterokaryotic hyphae. This follows the general hypothesis put forward by Pontecorvo (1958) to explain the parasexual phenomena in Aspergillus. It is maintained that a sorting out may produce a heterozygous diploid homokaryotic hypha, and that occasional mitotic crossing over and haploidization can occur in the diploid nuclei. If this is the case, a heterozygous diploid nucleus could, by a mechanism of mitotic crossing over, produce segregants and recombinants of linked genes.

Evidence of parasexual recombination was also found in this study (see Table I and Text-figure 2). The adenineless mutant used in the cross grown in pot 104 (Ad X29 (+) x X73 (-), a morphological mutant), was of the (+) mating type. However, both mating types were recovered. Also in Row 62 (Arg 244 (-) x Pan 191 (+), a double mutant), an argininelessand pantothenic acid mutant of mating type (+) was recovered. (Pantothenic acid mutant is linked with mating type locus (Hood, 1966)).

Row 824(1), a cross of isoleucine requiring V13 (+) and leucineless 394 (-) grew on minimal agar plates. Feulgen staining revealed a single nucleus. While it may be argued that mutations could cause these results, the frequency of these occurances is too great to be attributed to mutation only. It is concluded, therefore, that mitotic recombination has taken place in some of the cultures of U. hordei used in this study.

Sporidia obtained from part of the flag leaf from infected tiller A (Text-figure 2) showed an indefinite Bauch test (bisexual) and grew on minimal agar plates. Feulgen staining revealed several dikaryotic cells on one slide, the remainder of the slides showing uninucleate cells. This observation provides further evidence that dikaryotic nuclei may combine to form diploid sporidia. One would need to check this by inoculation of barley with these sporidia. (This was done (see Addendum Table II) with another suspected diploid, 824(3).) Since an infection was obtained it was concluded that it was a diploid.

Recovery of only one of the original nuclei (Table II) might be due to procedural shortcomings in shredding and shaking of dikaryotic mycelium. One, however, has to bear in mind that on shredding of the tissue, both nuclei might also be liberated. It may be that on culturing one of the two nuclei shows a selective advantage over the other and soon outnumbers the other. (This may be checked by placing equal amounts of these sporidia in a shake culture and growing these for several days after which they can be checked to determine whether there is a selective growth for one of the constituent nuclei.)

In conclusion, (through the use of biochemical mutants of U. hordei) evidence has been provided for the existence of parasexual recombination. It seems to be feasible to produce heterozygous diploids by making use of the sorting out process of the parasexual cycle which takes place after mitotic recombination. In addition to meiotic crossing over, mitotic recombination may become an important genetical tool in the mapping of the chromosomes of U. hordei in the same manner as it has been employed in chromosome studies of Aspergillus nidulans (Käfer, 1961; Pontecorvo and Roper, 1952).

SUMMARY

1. Haustoria occur in Ustilago hordei.

2. Sporidia can be recovered from meristematic tissue of barley.

3. There is evidence that Ustilago hordei makes use of the parasexual cycle (along with a normal sexual cycle). The parasexual cycle allows the organism to undergo mitotic segregation.

4. The dikaryotic phase of U. hordei may undergo haploidization. Diploidization by fusion of constituent nuclei may also occur. Both haploid and diploid nuclei may reside in the same host.

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Appendix 1. DNA assay

"The procedure for the determination of nucleic acids described below is based on the finding that nucleic acids can be separated from other tissue compounds by their preferential solubility in hot trichloroacetic acid. The isolated nucleic acids are then quantitated by means of colorimetric reactions involving the pentose components of nucleic acids (Schneider, 1957, p. 680). The procedure is as follows:

1. Samples of sporidia adjusted to a concentration of 10^7 sporidia per ml.
2. Removal of acid-soluble compounds:

   1 ml sporidia, 2.5 ml cold 10% TCA are centrifuged. Sediment is washed once with 2.5 ml cold TCA.
3. Removal of lipoidal compounds:

   Extract final sediment from above twice with 5 ml 95% ethanol. This extraction is centrifuged.
4. Removal of nucleic acids:

   Lipid free tissue is suspended in 1.3 ml water and 1.3 ml 10% TCA. This is heated for 15 min at 90° C (stir occasionally). This quantitatively splits the DNA and PNA from the tissue proteins. The latter (an insoluble residue) is centrifuged and washed with 2.5 ml cold 10% TCA.
5. Estimation of DNA:

   1 ml of the combined extracts of the above step are mixed with 2 ml diphenylamine and heated for 10 min in boiling water. The intensity of the blue color is read at 600 mμ (maximum absorption wave length).

"A standard curve is prepared relating optical density to micrograms of DNA-P, with purified DNA as the standard. The practice of using organic P content of DNA as the reference rather than DNA weight has the advantage that DNA samples of varying degrees of purity will yield the same curve when diphenylamine color intensity is plotted against DNA-P."

Appendix 2. Bauch (mating type) test

It has long been established that many of the smut fungi are heterothallic. Before normal infection is possible a fusion of two monosporidial lines of opposite mating type is necessary. This concept was first shown by Kniep (1919) and later reported by Bauch (1927), Dickinson (1927) and others.

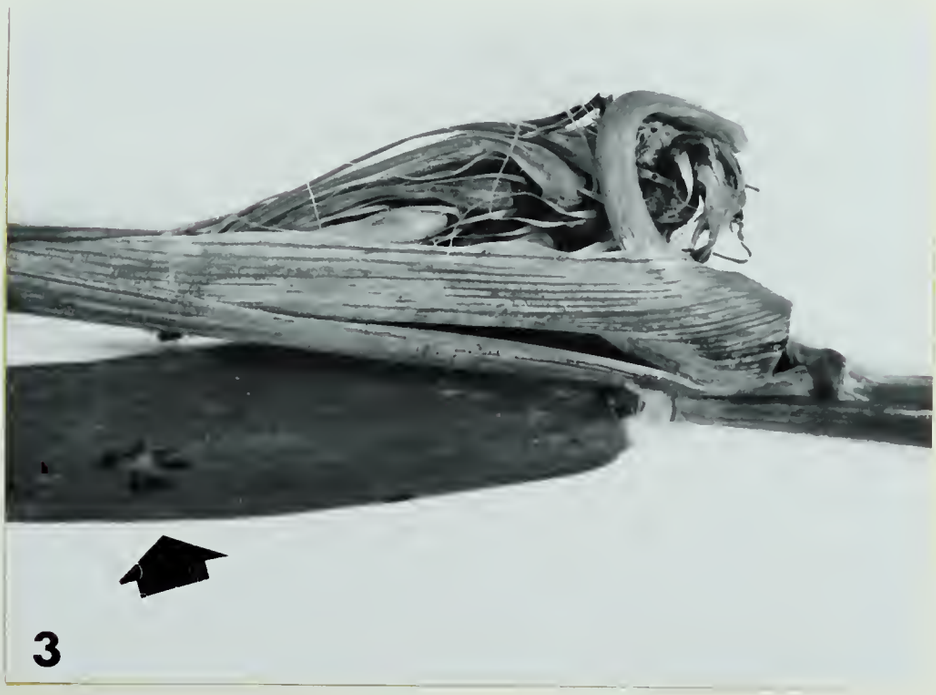
In order to establish compatible relationships between pairs of monosporidial lines in this study a test based on sporidial fusion and the subsequent growth of infective hyphae was used. Sporidia from an unsexed monosporidial line were placed in pairs on special agar plates (20 ml Vogel's salt solution, 2 g dextrose, 20 g Difco bacto agar per liter of distilled water). These were then mixed with known strains, i.e. mating type (+) and (-). These were then incubated at 22^{o} C for 8 hr. Aerial hyphae consisting of a cotton-like, white mycelial growth (Suchsfaden) were used as an indication of compatibility.

PLATE 1

Figs. 1, 2, 3. Spikes of barley infected with *U. hordei* illustrating typical deformities caused by the disease. Note Fig. 3 (arrow) masses of teliospores on the epidermis of a flag leaf.

1

2



3

PLATE 2

Figs. 4 and 5. Heavily smutted spikes. Note smutted stem (Fig. 4), and crinkled leaves, indicative of infection (Fig. 5).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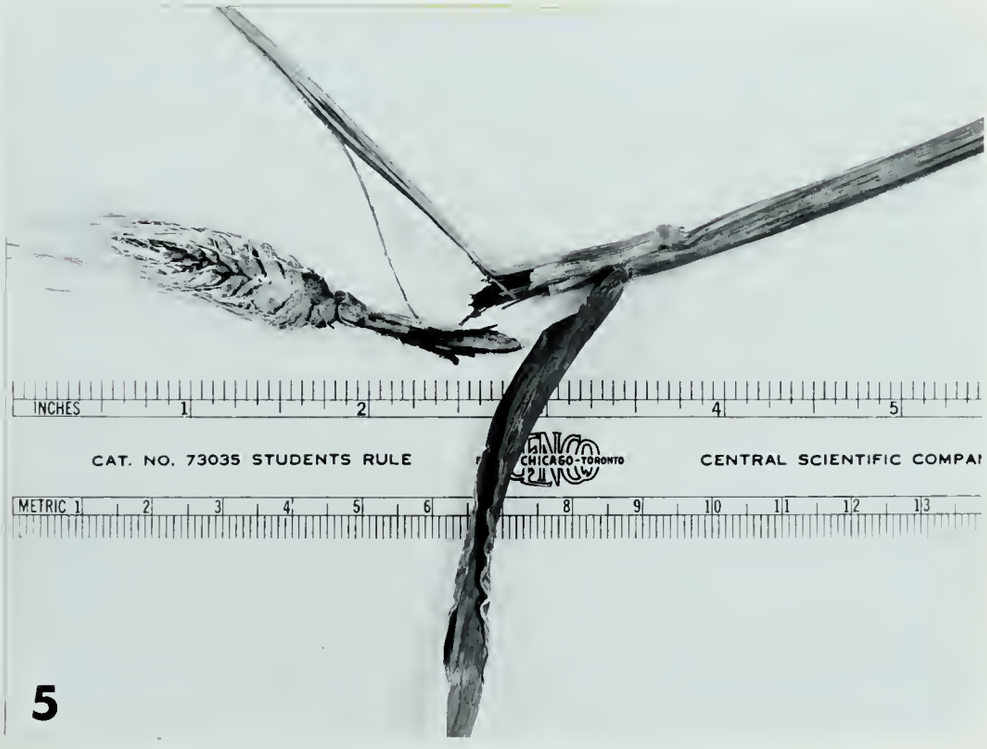
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PLATE 3

Fig. 6. Spikes showing varying degrees of infection.

Fig. 7. Completely smutted spike. The caryopsides although of normal form were completely filled with teliospores.


6
1
2
3
4
5

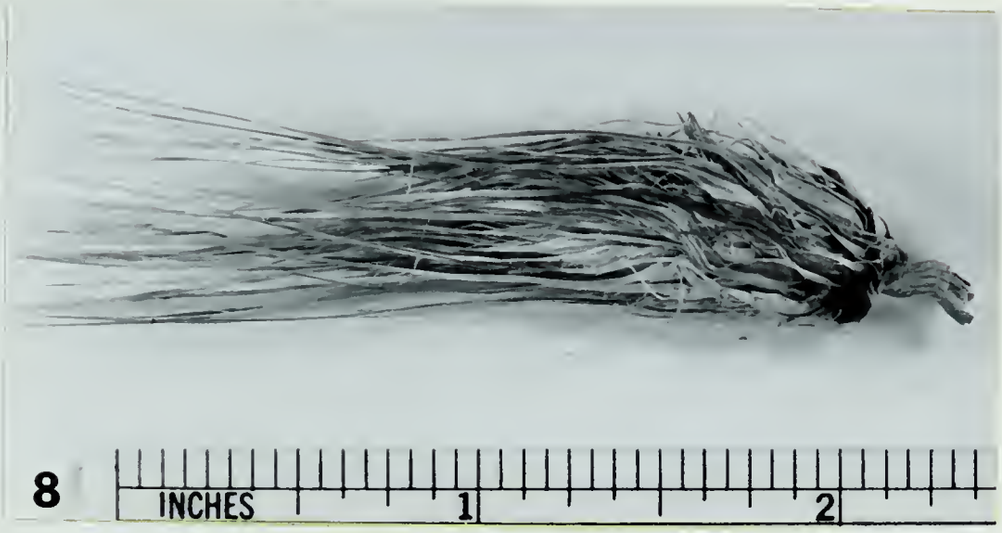

7
1
2

PLATE 4

Fig. 8. Clubbed spike showing stunting effect and marked reduction in size of the teliospore mass.

Fig. 9. Infected spike with a partial clubbing effect. Teliospore masses are visible on the lower portion of the spike. The spike also serves to illustrate the difficulty smutted spikes have in emerging from the flag leaf.

Fig. 10. Normal spike and clubbed spike with moderate infection.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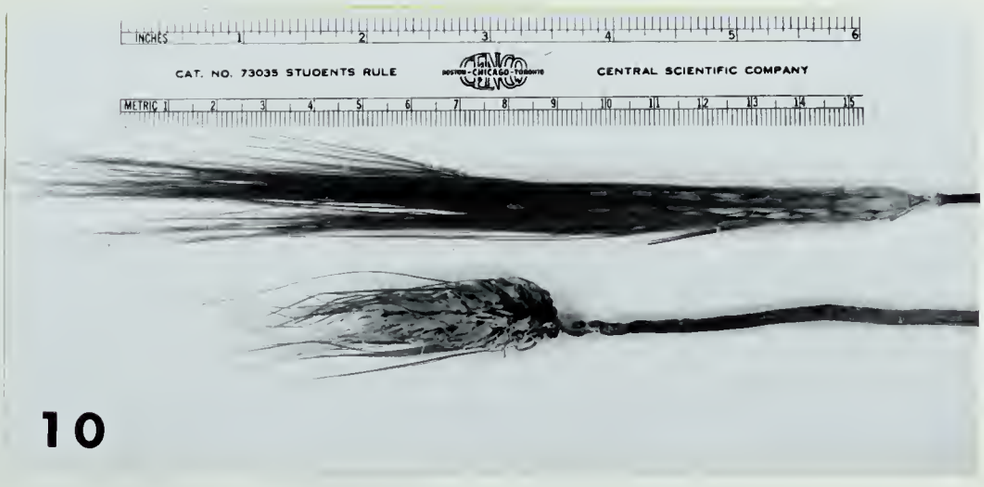
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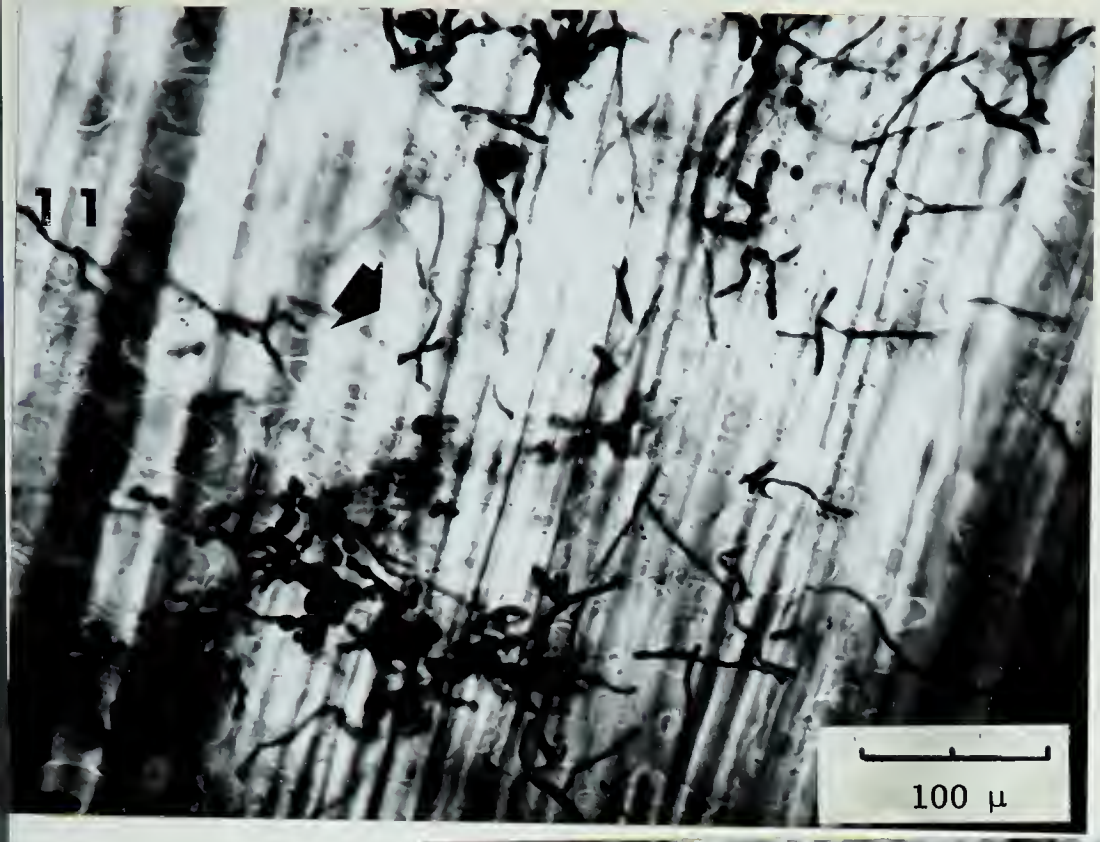
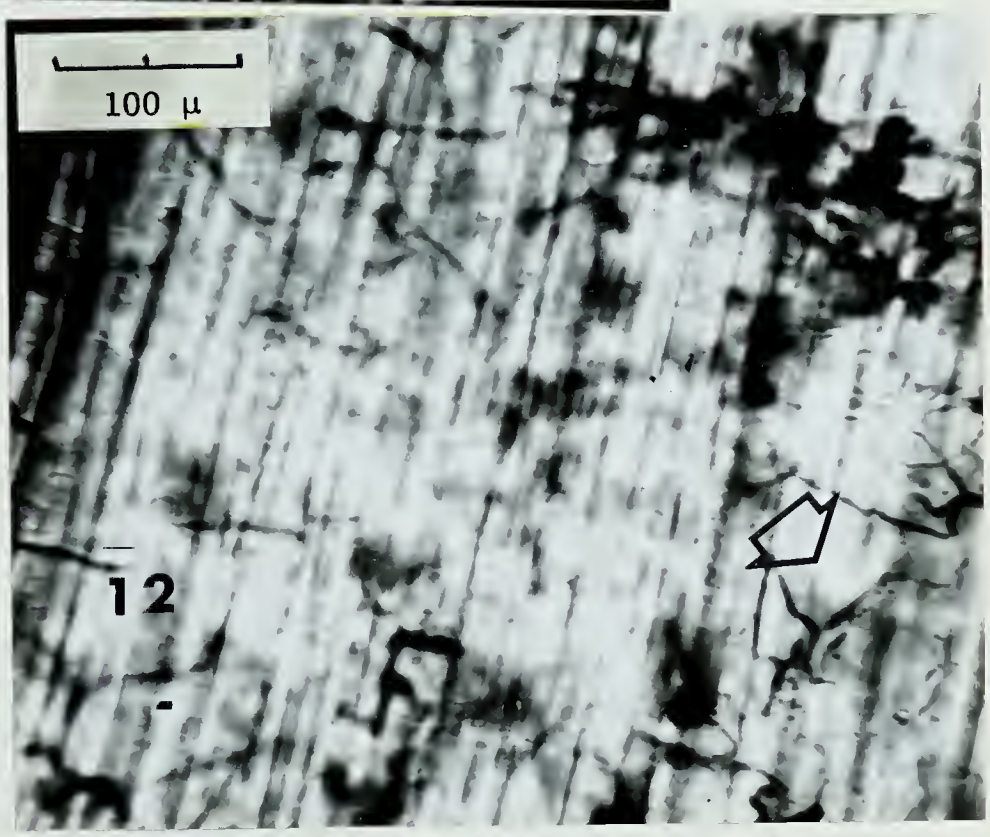
PLATE 5

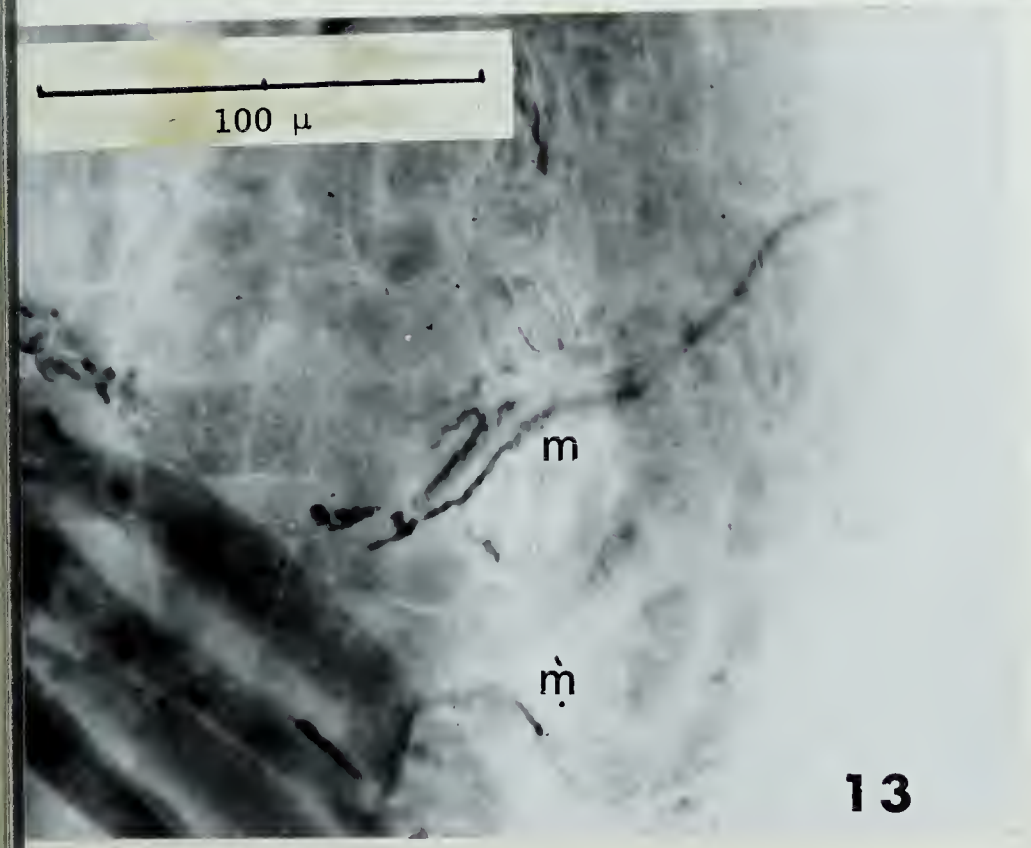
Figs. 11 and 12. Squash preparations of germinating sporidia on the hull of an inoculated barley seed after 36 hrs incubation. Arrows indicate mycelial penetration of the hull.

Fig. 13. Squash preparation of an infected embryo showing several strands of mycelium.

m = mycelium







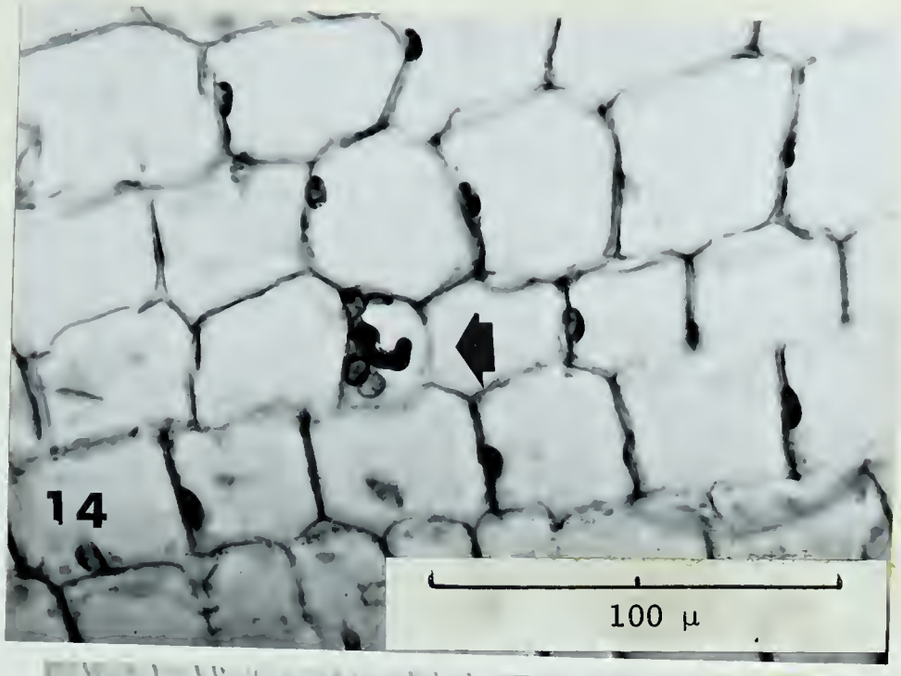
13

PLATE 6

Fig. 14. Haustoria (arrow) in meristematic tissue of node of an infected tiller.

Fig. 15. Longitudinal section illustrating haustoria (arrow) situated in parenchymatous tissue of an infected node. Note hyphal invasion (h) of fiber tissue of the stem.

Fig. 16. Haustoria in parenchymatous tissue (arrow).


14
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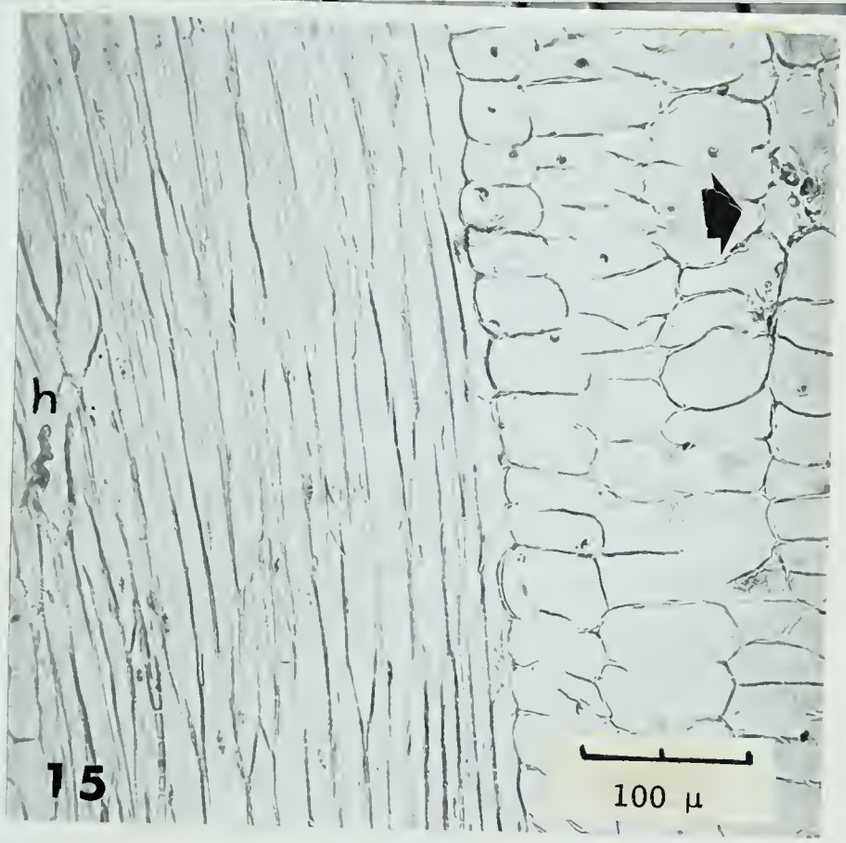
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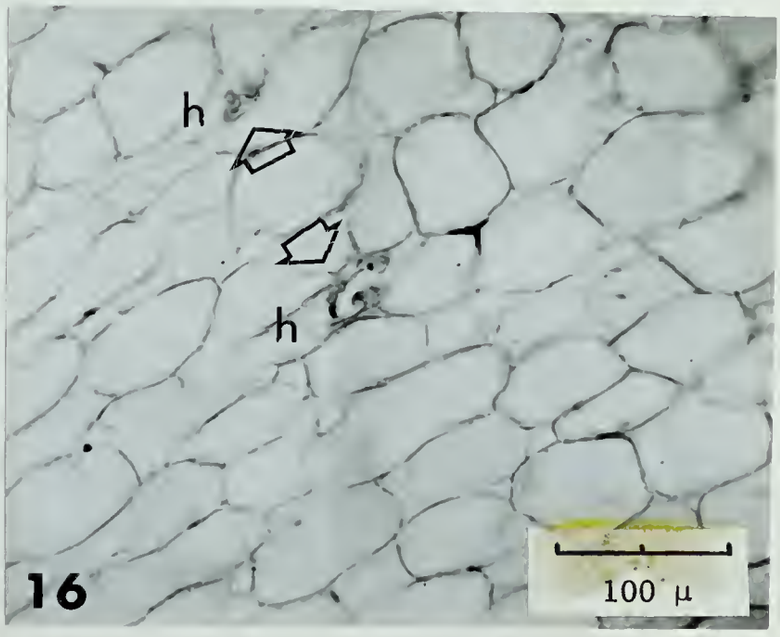
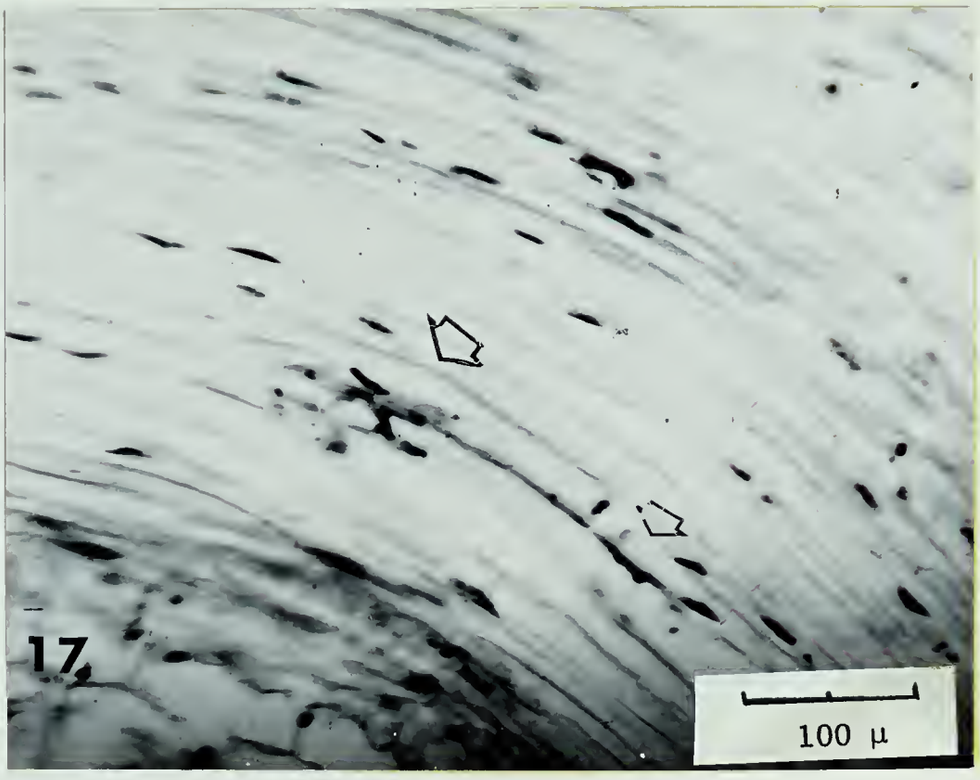

h
h
16
100 µ

PLATE 7

Fig. 17. Transverse section of the third node of an infected tiller showing mycelia fragments (arrows).

Fig. 18. Longitudinal section of an infected node showing intercellular mycelium (arrow).

h = haustoria


17
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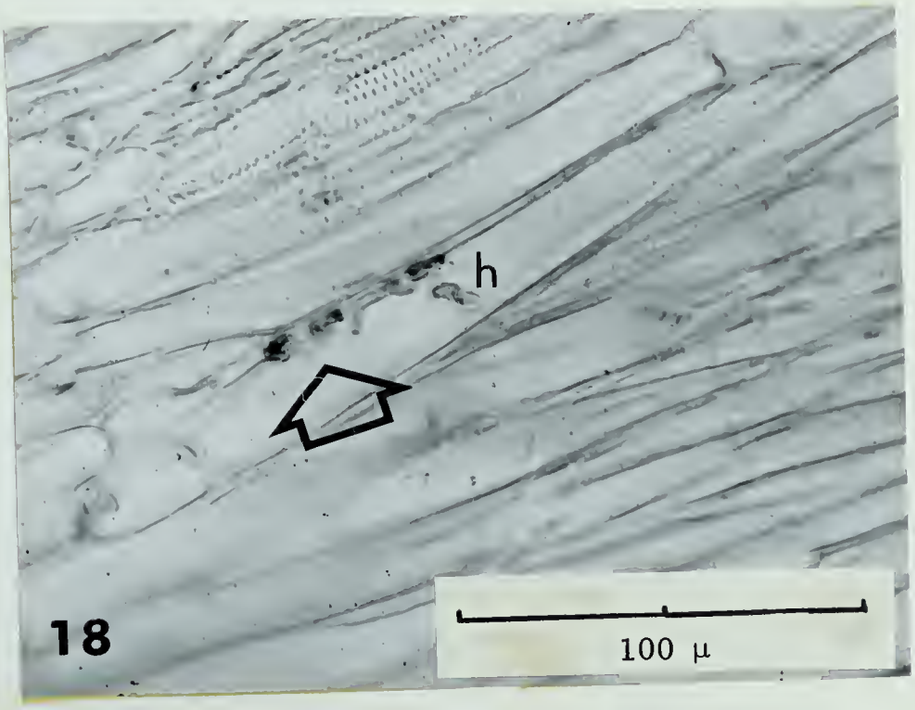

h
18
100 μ

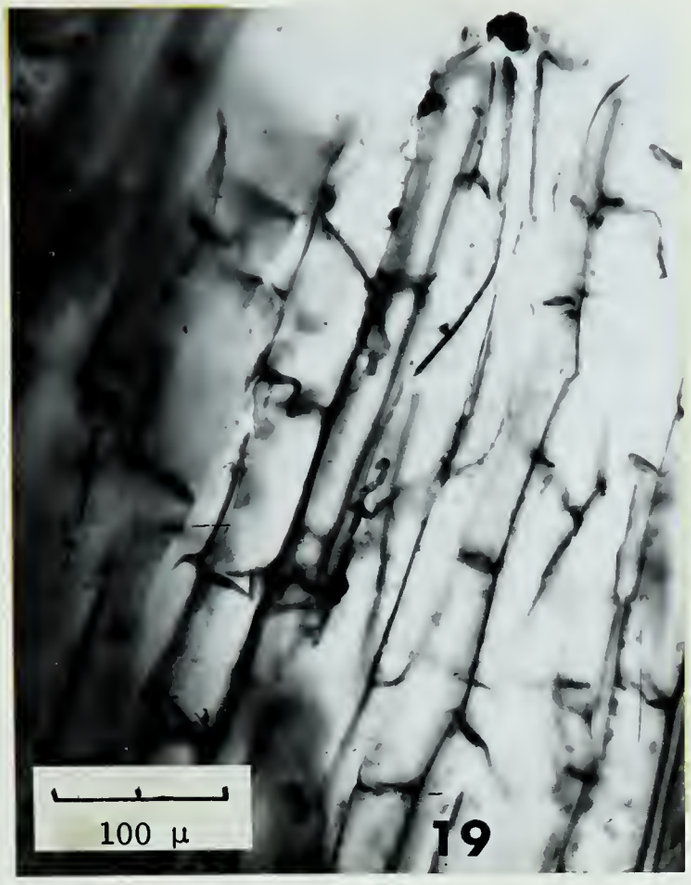
PLATE 8

Fig. 19. Longitudinal section of an infected tiller internode showing long, slender mycelium in the phloem.

Fig. 20. Enlarged portion of Fig. 19 showing mycelial detail.

Fig. 21. Longitudinal section view of the outer portion of an infected internode showing xylem tissue (xy) and short, thick, mycelial sections (arrows) in the meristematic tissue.

Fig. 22. Longitudinal section of an infected internode showing intercellular mycelium (i) and intracellular mycelium (arrow).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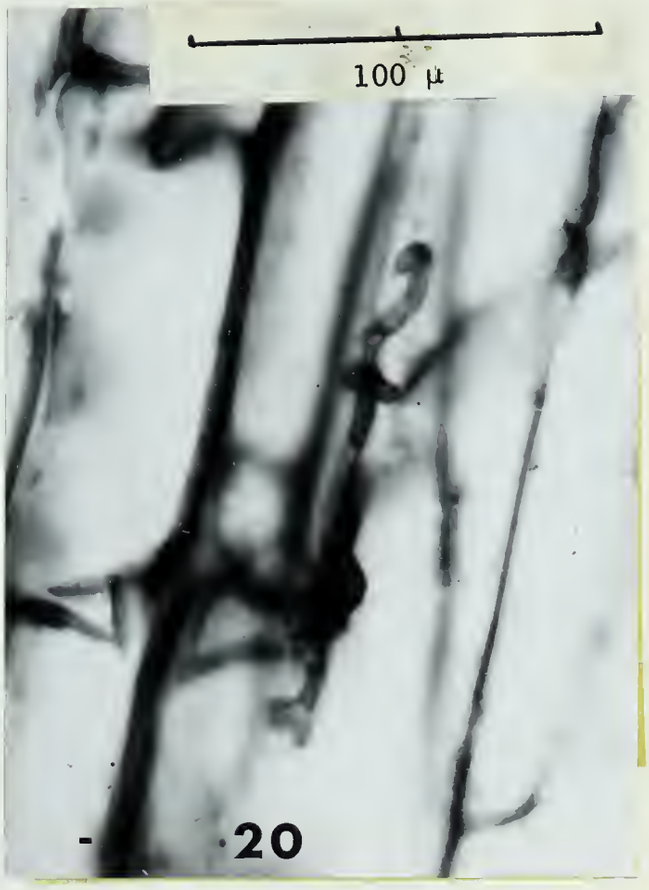
19


100 μ
20


X
Y
21
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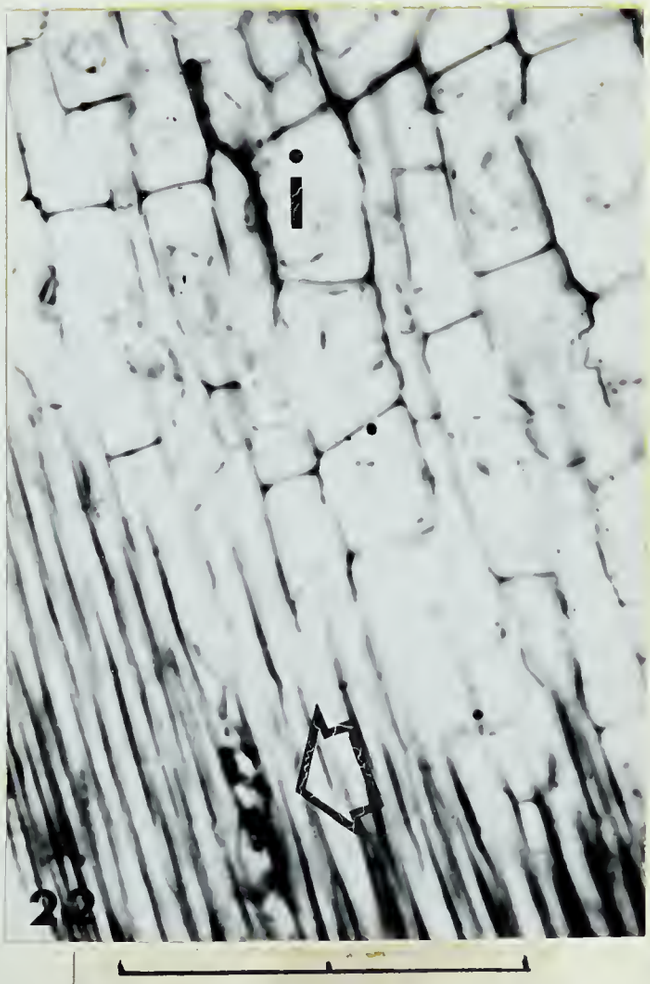
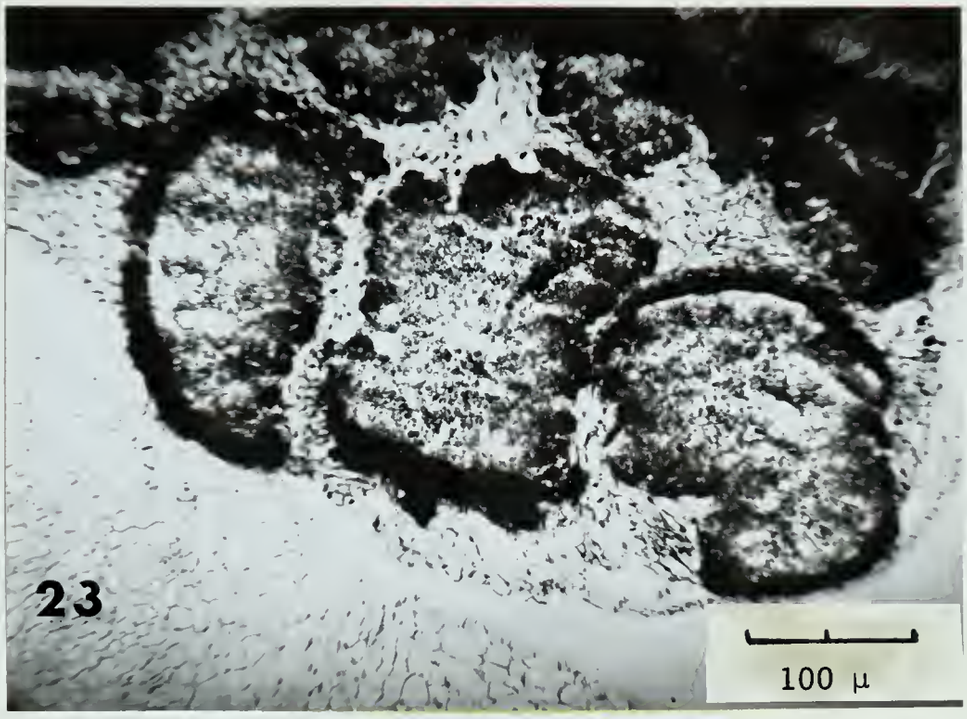

i
22
100 μ

PLATE 9

Fig. 23. Longitudinal section of an infected spike. Masses of teliospores are visible in the florets.

Fig. 24. Enlarged view of a floret illustrating masses of mature teliospores.


23
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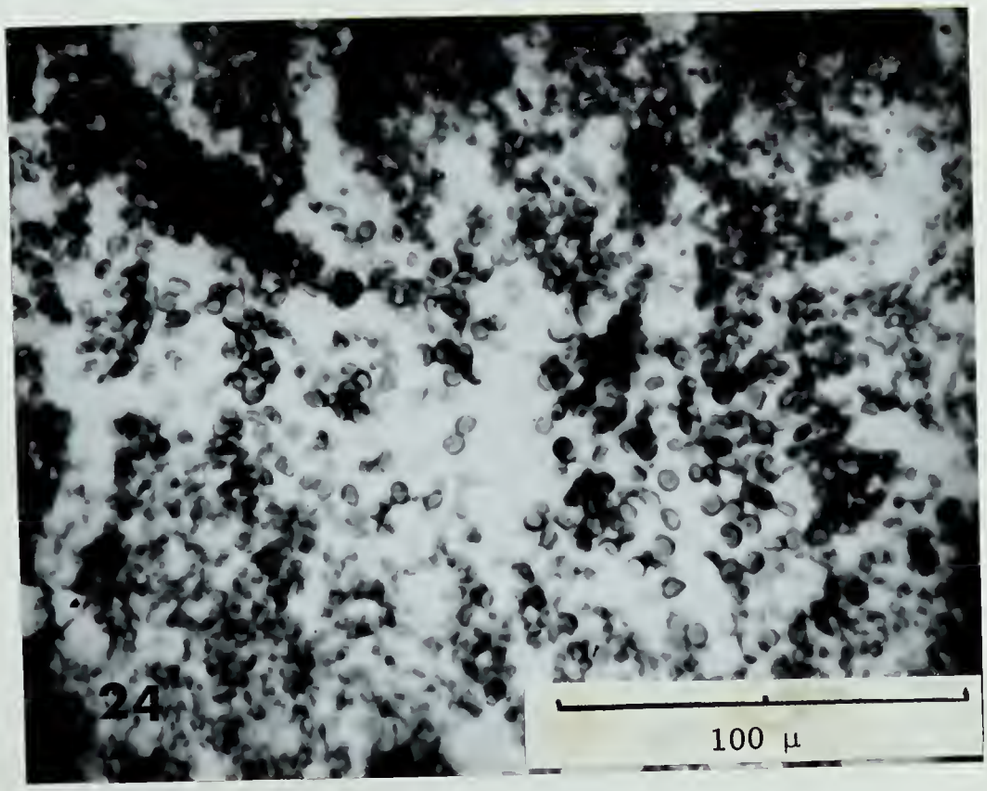
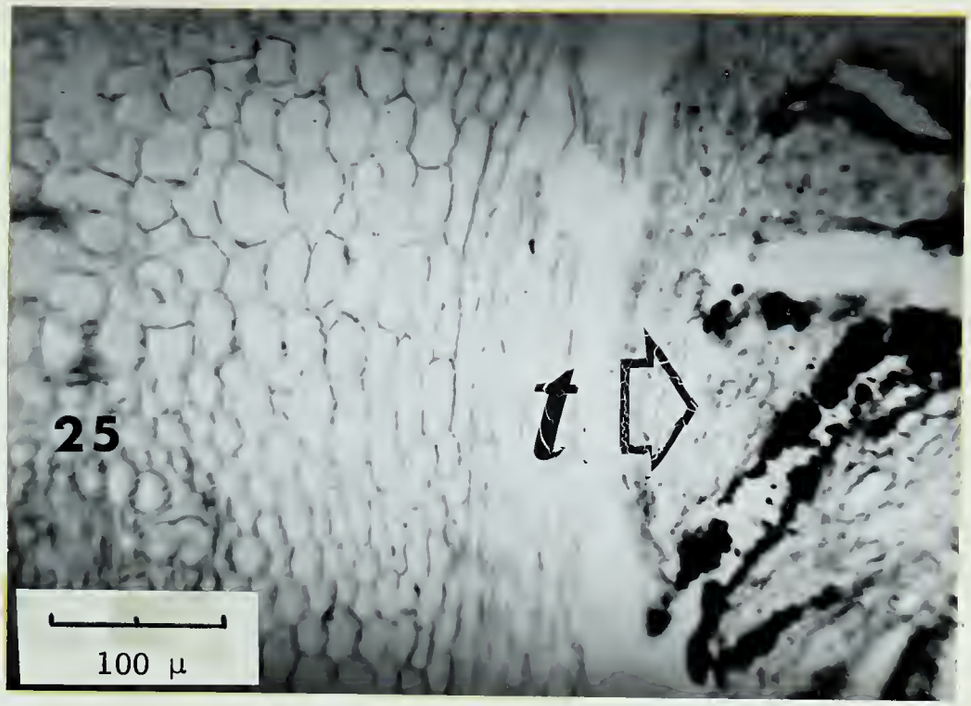

24
100 μ

PLATE 10

Fig. 25. Transverse section of the fourth node of an infected tiller showing pockets of teliospores (t).

Fig. 26. Enlarged view of a spore pocket (Fig. 25) showing its relationship to the phloem and xylem tissue.

Fig. 27. Enlarged view of a spore pocket showing teliospore detail.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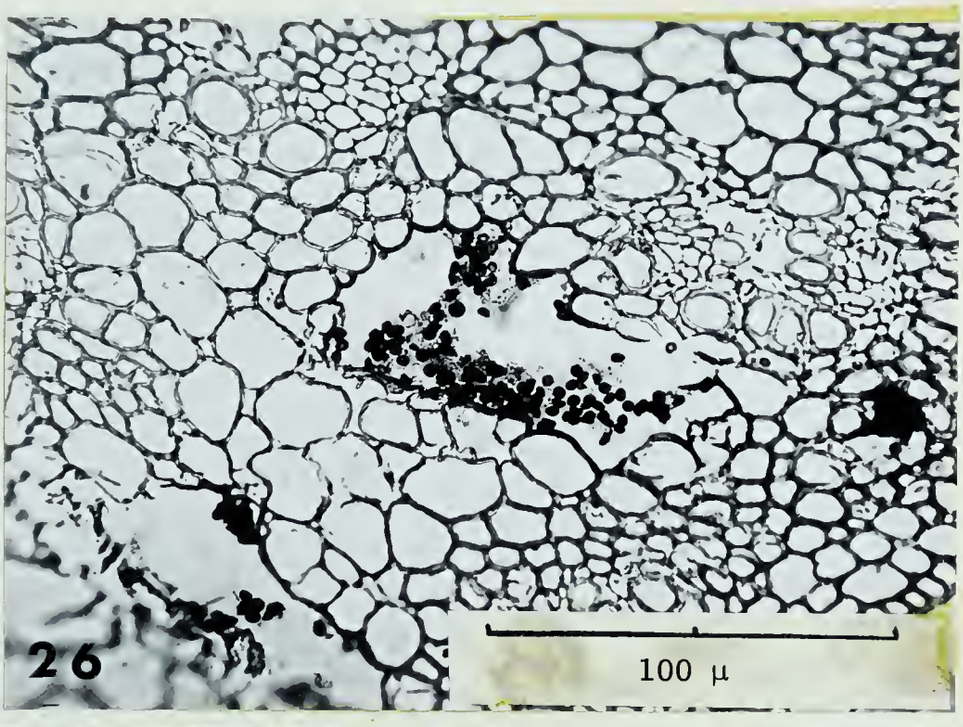
t
100 μ


26
100 μ

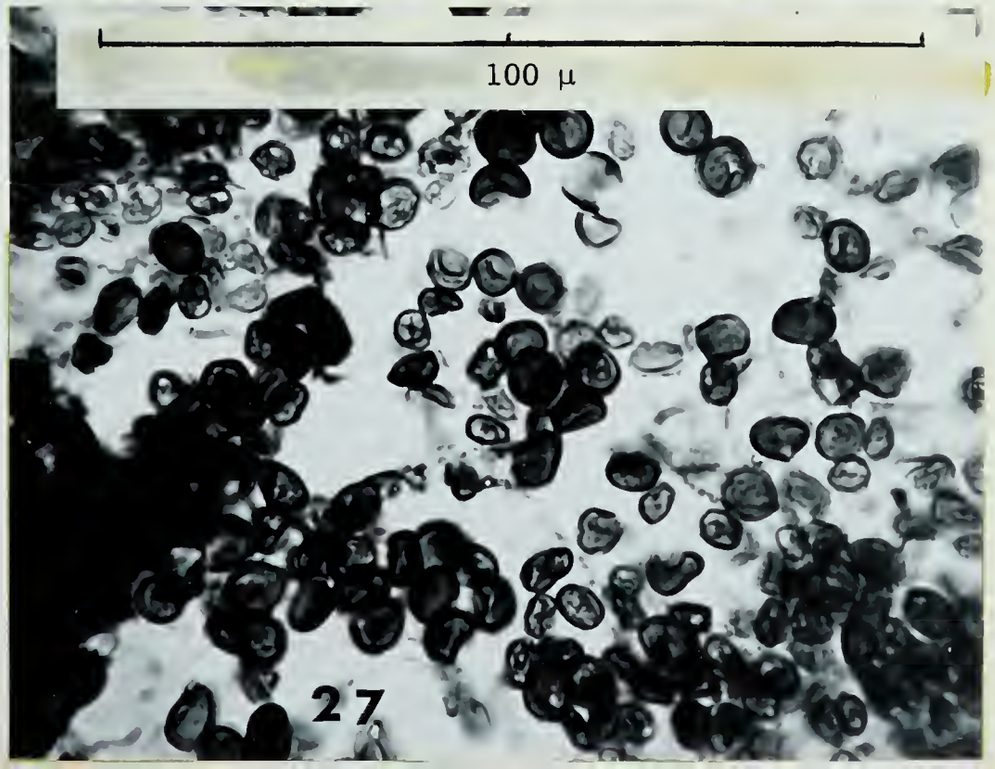
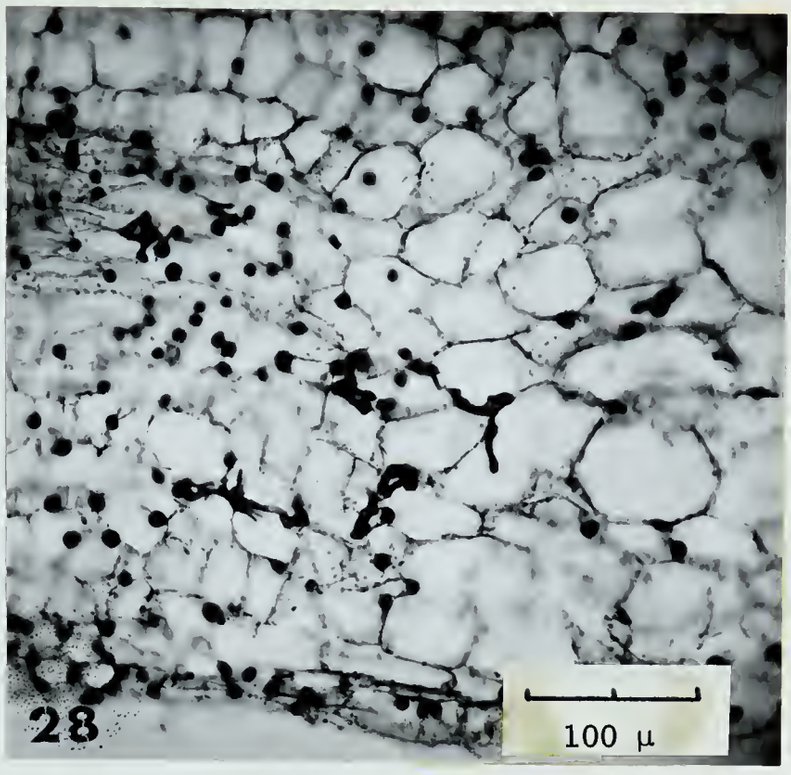

100 μ
27

PLATE 11

Figs. 28 and 29. Teliospores were frequently found in spike nodes in conjunction with short, thick, mycelial fragments (myf). Spores are formed when these shorten, thicken, segment, round off and form a teliospore wall.


28
100 μ

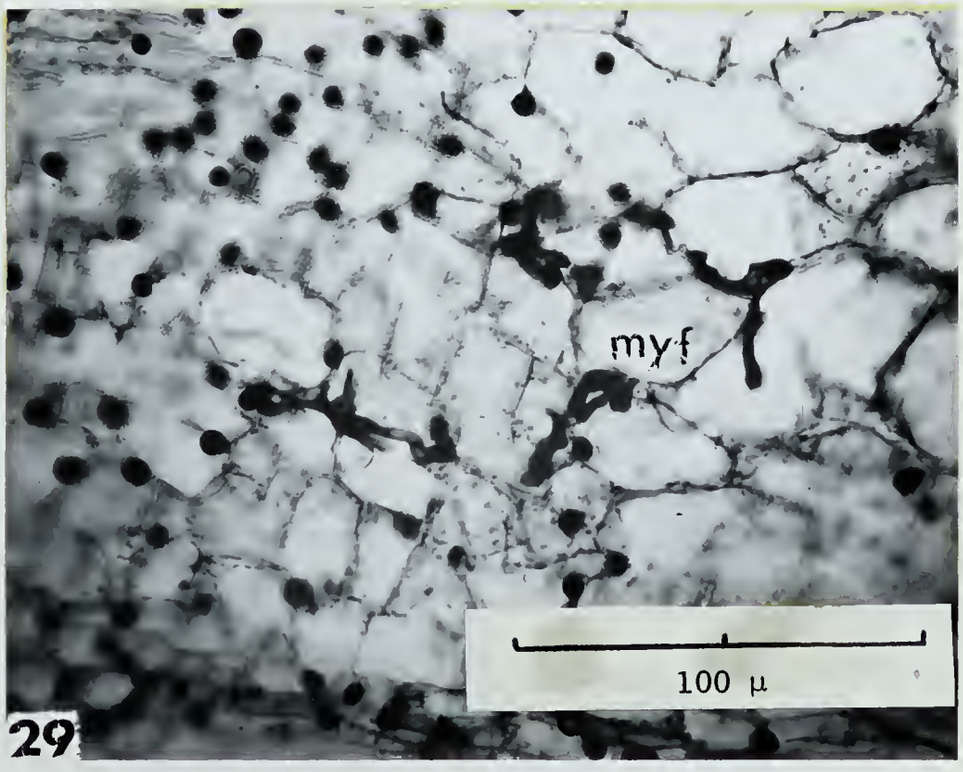
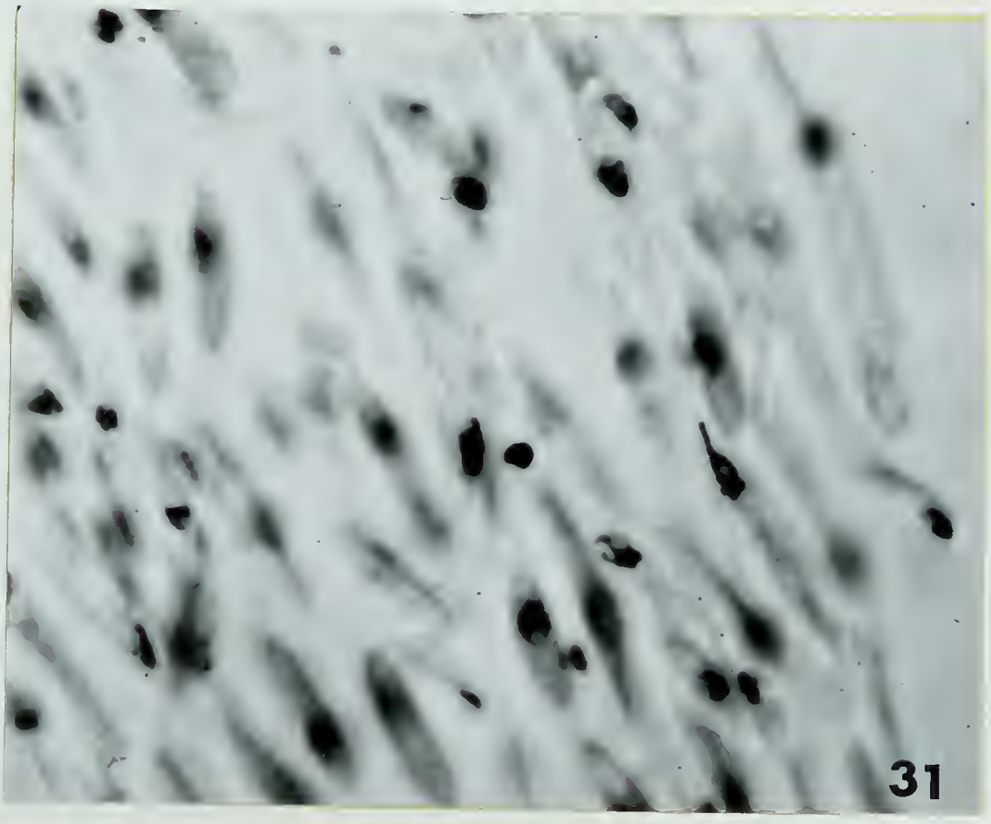

myf
100 μ
29

PLATE 12

Figs. 30 and 31. Sporidia of *Ustilago hordei* (culture 824(3)) stained with Feulgen.


30


31